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[Translation]

Japanese Patent Application Laid-Open Publication (Kokai)

No. Hei 6-253854

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Application Number	Hei 5-63515
Date of Application	February 26, 1993
Applicant	Yamasa Shoyu K.K.
Inventors	Noguchi T. & other 3 persons

[Title of the Invention]

Process for Preparation of Nucleoside-Phosphorylase by  
Recombinant DNA Technique

[Synopsis]

[Object]

The invention has an object of preparing a large amount of nucleoside-phosphorylase by the recombinant DNA technique intending to obtain possibility of efficient synthesis of a nucleoside analogues using nucleoside-phosphorylase.

[Constitution]

The present invention relates to a process for preparation of DNA molecules containing a nucleoside-phosphorylase-structured gene, to preparation of a nucleoside-phosphorylase by the recombinant DNA technique using the same, and to a process for preparation of nucleoside in which the cultivated product, the microbe biomass or the processed product thereof obtained by this method method is used as the enzyme source.

[What is claimed is]

[Claim 1]

A DNA molecule containing a purine nucleoside-phosphorylase-structured gene which codes the amino acid sequence of following formula (I) derived from thermophilic bacteria belonging to bacillus genus

[Formula I]

10	20
MetAsnArgThrAlaIleGluGlnAlaAlaGlnPheLeuLysGluLysPheProThrSer	
30	40
ProGlnIleGlyLueIleLeuGlySerGlyLeuGlyValLeuAlaAspGluIleGluGln	
50	60
AlaIleLysIleProTyrSerAspIleProAsnPheProValSerThrValGluGlyHis	
70	80
AlaGlyGlnLeuValTyrGlyGlnLeuGluGlyAlaThrValValMetGlnGlyArg	
90	100
PheHisTyrTyrGluGlyTyrSerPheAspLysValThrPheProValArgValMetLys	
110	120
AlaLeuGlyValGluGlnLeuIleValThrAsnAlaAlaGlyGlyValAsnGluSerPhe	
130	140
GluProGlyAspLeuMetIleIleSerAspHisIleAsnAsnMetGlyGlyAsnProLeu	
150	160
IleGlyProAsnAspSerAlaLeuGlyValArgPheProAspMetSerGluAlaTyrSer	
170	180
LysArgLeuArgGlnLeuAlaLysAspValAlaAsnAspIleGlyLeuArgValArgGlu	
190	200
GlyValTyrValAlaAsnThrGlyProAlaTyrGluThrProAlaGluIleArgMetIle	
210	220
ArgValMetGlyGlyAspAlaValGlyMetSerThrValProGluValIleValAlaArg	
230	240
HisAlaGlyMetGluValLeuGlyIleSerCysIleSerAsnMetAlaAlaGlyIleLeu	
250	260
AspGlnProLeuThrHisAspGluValIleGluThrThrGluLysValLysAlaAspPhe	
270	
LeuArgPheValLysAlaIleValArgAsnMetAlaLysAsn	

(I)

## [Claim 2]

The DNA molecule according to Claim 1 which contains an SD sequence in the upstream of the purine nucleoside-phosphorylase-structured gene

## [Claim 3]

A DNA molecule containing pyrimidine nucleoside-phosphorylase-structured gene which codes the amino acid sequence the following formula (II) derived from thermophilic bacteria belonging to bacillus genus.

## [Formula 2]

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10	20
MetArgMetValAspLeuIleGluLysLysArgAspGlyHisAlaLeuThrLysGluGlu	
30	40
IleGlnPheIleIleGluGlyTyrThrLysGlyAspIleProAspTyrGlnMetSerAla	
50	60
LeuAlaMetAlaIlePhePheArgGlyMetAsnGluGluGluThrAlaGluLeuThrMet	
70	80
AlaMetValHisSerGlyAspThrIleAspLeuSerArgIleGluGlyIleLysValAsp	
90	100
LysHisSerThrGlyGlyValGlyAspThrThrThrLeuValLeuGlyProLeuValAla	
110	120
SerValGlyValProValAlaLysMetSerGlyArgGlyLeuGlyHisThrGlyGlyThr	
130	140
IleAspLysLeuGluSerValProGlyPheHisValGluIleThrAsnAspGluPheIle	
150	160
AspLeuValAsnLysAsnLysIleAlaValValGlyGlnSerGlyAsnLeuThrProAla	
170	180
AspLysLysLeuTyrAlaLeuArgAspValThrAlaThrValAsnSerIleProLeuIle	
190	200
AlaSerSerIleMetSerLysLysIleAlaAlaGlyAlaAspAlaIleValLeuAspVal	
210	220
LysThrGlyValGlyAlaPheMetLysAspLeuAsnAspAlaLysAlaLeuAlaLysAla	
230	240
MetValAspIleGlyAsnArgValGlyArgLysThrMetAlaIleIleSerAspMetSer	

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[式3]

[Formula 3]

[Formula 3]

250	260
GlnProLeuGlyTyrAlaIleGlyAsnAlaLeuGluValLysGluAlaIleAspThrLeu	
270	280
LysGlyGluGlyProGluAspPheGlnGluLeuCysLeuValLeuGlySerHisMetVal	
290	300
TyrLeuAlaGluLysAlaSerSerLeuGluGluAlaArgHisMetLeuGluLysAlaMet	
310	320
LysAspGlySerAlaLeuGlnThrPheLysThrPheLeuAlaAlaGlnGlyGlyAspAla	
330	340
SerValValAspAspProSerLysLeuProGlnAlaLysTyrIleIleGluLeuGluAla	
350	360
LysGluAspGlyTyrValSerGluIleValAlaAspAlaValGlyThrAlaAlaMetTrp	
370	380
LeuGlyAlaGlyArgAlaThrLysGluSerThrIleAspLeuAlaValGlyLeuValLeu	
390	400
ArgLysLysValGlyAspAlaValLysLysGlyGluSerLeuValThrIleTyrSerAsn	
410	420
ArgGluGlnValAspAspValLysGlnLysLeuTyrGluAsnIleArgIleSerAlaThr	
430	
ProValGlnAlaProThrLeuIleTyrAspLysIleSer	

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[Claim 4]

The DNA molecule according to Claim 3 which contains an SD sequence in the upstream of pyrimidine nucleoside, phosphorylase-structured gene.

[Claim 5]

A recombinant vector built up by integration of the DNA molecule according to Claim 1 and/or Claim 3 in the downstream of the controlling signal for expression of a vector which is capable of replication in the cell.

[Claim 6]

The recombinant vector according to Claim 5 which

contains at least a promoter the expression-controlling signal of which functions in Escherichia coli.

[Claim 7]

A cultivated product which contains a microbe biomass which retains nucleoside-phosphorylase derived from thermophilic bacteria in which the relevant enzyme has been generated by cultivation of a transformant transformed by the recombinant vector according to Claim 5

[Claim 8]

A microbe biomass which retains nucleoside-phosphorylase derived from thermophilic bacteria isolated from the cultivated product according to Claim 7, or a processed product thereof.

[Claim 9]

A process for preparation of nucleoside characterized in that, in the process in which a base donor, a sugar-residue donor and a phosphoric acid donor are reacted using an enzyme preparation which contains a nucleoside-phosphorylase to form an N-glicoside linkage between the base segment of the base donor and the sugar segment of the sugar-residue donor, the product according to Claim 7 or Claim 8 is used as the enzyme preparation containing a nucleoside-phosphorylase.

[Detailed description of the invention]

[0001]

[Field of application in industry]

The present invention relates to a process for preparation of nucleoside-phosphorylase derived from thermophilic bacteria belonging to bacillus genus by the

technique of recombined DNA and to application of the enzyme obtained through such a process to production of nucleoside.

[0002]

[Prior art]

Chemotherapeutic agents of nucleic acid type are used in various use fields such as antineoplasma, immunosuppression, antiviral and so forth. Also, the antiviral action of nucleoside analogues is attracting attention with epidemic of AIDS in recent years to bring about synthesis of various nucleoside analogues which are subjected to examination of the antiviral activity thereof ("Kagaku-to-Seibutsu", Vol. 27, No. 6, p.356~p.366). Although many of these nucleoside analogues have been hitherto synthesized chemically, in the currency of elucidation of the fact that various nucleoside analogues can be efficiently synthesized by utilizing nucleoside-phosphorylase derived from microorganisms, utilization of such an enzyme has come to be regarded as an important means for synthesis of a nucleoside analogue ("Hakko to Kogyo", Vol. 39, No. 10, p.927~p.937).

[0003]

In general, microorganisms are advantageous as the source for preparation of enzymes from the viewpoint of operability and economy. It has been confirmed that nucleoside-phosphorylases exist in various organisms including animals, microorganisms etc. and the enzymological characteristics of some of them have been reported after isolation and refining thereof. For instance, existence of nucleoside-phosphorylase has been confirmed also with respect to *Bacillus*



stearothermophilus as a kind of thermophilic bacteria belonging to bacillus genus. That is to say, both of purine nucleoside-phosphorylase (E.C.2,4,2,1.) and pyrimidine nucleoside-phosphorylase (E.C.2,4,2,2.) have been isolated from such microorganisms and refined for reporting various characteristics thereof (J. Biol. Chem., 244, 3691 (1969); Agric. Biol. Chem., 53, 2205 (1989); and Agric. Biol. Chem., 53, 3219 (1989)). Synthesis of a nucleoside analogue using such enzymes have been also reported (Agric. Biol. Chem., 53, 197-202 (1989); Japanese Patent Laid-Open Publication Sho 56-166199; Japanese Patent Laid-Open Publication Sho 56-164793; and Japanese Patent Laid-Open Publication Hei 1-320995).

[0004]

Yamanouchi et al. have found out Bacillus stearothermophilus, TH6-2 strain which contains nucleoside-phosphorylases (purine nucleoside-phosphorylase and pyrimidine nucleoside-phosphorylase) having thermotolerance and high specific activity from thermophilic bacteria belonging to bacillus genus and have succeeded in isolation of a nucleoside-phosphorylase from this microbe strain (International Patent Laid-Open Publication WO90/10080; "Nippon Nogei Kagaku Kai-shi", Vol. 63, No. 3, (Lecture Abstracts for 1989 Convention), p.283).

[0005]

[Problems to be solved by the invention]

Although the above enzymes found out by Yamanouchi et al. are extremely excellent enzymes, in the cases of using the microbe biomass per se of the microorganisms as the enzyme source for nucleoside preparation, the enzyme is released in the

reaction medium due to autolysis accompanied with spore formation inducing disadvantages that continuous synthesis of nucleoside is unfavorably influenced or that synthesis or purification of nucleoside is hindered by flowing out of the microbe biomass accompanied with bacteriolysis. Furthermore, it is necessary to continually recognize that, as a common problem in the cases of using a microbe biomass of microorganisms as an enzyme source, there exists a problem that various enzymes included in the microbe biomass induce side reactions such as disintegration of the stroma or the product bringing about decrease of the product yield.

[0006]

Although a method of using a preparation of purified enzyme as the enzyme source has been taken into consideration in order to solve the problems in the method of using such a microbe biomass of microorganisms as an enzyme source, it has been a matter of difficulty in fact to collect the nucleoside-phosphorylase from the microbe biomass in a high yield since bacillus stearothermophilus has decomposing activity against protein, nucleoside-phosphorylase is produced only in little amount and the refining operation of the enzyme is troublesome.

[0007]

Yamanouchi has carried out cloning of Escherichia coli with a DNA fragment containing a gene for coding the nucleoside-phosphorylase derived from bacillus stearothermophilus as the first step for overcoming the above problem to find out a fact that the relevant enzyme can be produced by Escherichia coli. (Japanese Patent Laid-Open Publication Hei 4-4882).

However, the production amount of nucleoside-phosphorylase by the recombinant *Escherichia coli* created by the method is equal to or less than the production amount thereof by the thermophilic bacteria so that, even if such bacteria are used as the enzyme source for nucleoside preparation, such a measure has been by no means bearable in practical application.

[0008]

Means to solve the problem]

The inventors have carried out extensive studies with an object of solving the above problem consequently to succeed in analyzing the primary structure of the DNA for coding the nucleoside phosphorylase derived from *Bacillus stearothermophilus* and, on the basis of the result of this analysis, in mass production of the enzyme by *Escherichia coli* applying the recombinant DNA technique thus to complete the present invention.

[0009]

That is to say, the present invention relates to DNA molecules which contain a purine nucleoside-phosphorylase-structured gene for coding the amino acid sequence of the following Formula (I) derived from thermophilic bacteria belonging to *Bacillus* genus.

[0010]

[Formula 4]

10	20
MetAsnArgThrAlaIleGluGlnAlaAlaGlnPheLeuLysGluLysPheProThrSer	
30	40
ProGlnIleGlyLueIleLeuGlySerGlyLeuGlyValLeuAlaAspGluIleGluGln	
50	60
AlaIleLysIleProTyrSerAspIleProAsnPheProValSerThrValGluGlyHis	
70	80
AlaGlyGlnLeuValTyrGlyGlnLeuGluGlyAlaThrValValValMetGlnGlyArg	
90	100
PheHisTyrTyrGluGlyTyrSerPheAspLysValThrPheProValArgValMetLys	
110	120
AlaLeuGlyValGluGlnLeuIleValThrAsnAlaAlaGlyGlyValAsnGluSerPhe	
130	140
GluProGlyAspLeuMetIleIleSerAspHisIleAsnAsnMetGlyGlyAsnProLeu	
150	160
IleGlyProAsnAspSerAlaLeuGlyValArgPheProAspMetSerGluAlaTyrSer	
170	180
LysArgLeuArgGlnLeuAlaLysAspValAlaAsnAspIleGlyLeuArgValArgGlu	
190	200
GlyValTyrValAlaAsnThrGlyProAlaTyrGluThrProAlaGluIleArgMetIle	
210	220
ArgValMetGlyGlyAspAlaValGlyMetSerThrValProGluValIleValAlaArg	
230	240
HisAlaGlyMetGluValLeuGlyIleSerCysIleSerAsnMetAlaAlaGlyIleLeu	
250	260
AspGlnProLeuThrHisAspGluValIleGluThrThrGluLysValLysAlaAspPhe	
270	
LeuArgPheValLysAlaIleValArgAsnMetAlaLysAsn	

(I)

[0011]

Further, the present invention relates to DNA molecules which contains a pyrimidine nucleoside-phosphorylase-structured gene for coding the amino acid sequence of the following Formula (II) derived from thermophilic bacteria belonging to bacillus genus.

[0012]

(7)

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[Formula 5] 11

12

10	20
MetArgMetValAspLeuIleGluLysLysArgAspGlyHisAlaLeuThrLysGluGlu	
30	40
IleGlnPheIleIleGluGlyTyrThrLysGlyAspIleProAspTyrGlnMetSerAla	
50	60
LeuAlaMetAlaIlePhePheArgGlyMetAsnGluGluGluThrAlaGluLeuThrMet	
70	80
AlaMetValHisSerGlyAspThrIleAspLeuSerArgIleGluGlyIleLysValAsp	
90	100
LysHisSerThrGlyGlyValGlyAspThrThrThrLeuValLeuGlyProLeuValAla	
110	120
SerValGlyValProValAlaLysMetSerGlyArgGlyLeuGlyHisThrGlyGlyThr	
130	140
IleAspLysLeuGluSerValProGlyPheHisValGluIleThrAsnAspGluPheIle	
150	160
AspLeuValAsnLysAsnLysIleAlaValValGlyGlnSerGlyAsnLeuThrProAla	
170	180
AspLysLysLeuTyrAlaLeuArgAspValThrAlaThrValAsnSerIleProLeuIle	
190	200
AlaSerSerIleMetSerLysLysIleAlaAlaGlyAlaAspAlaIleValLeuAspVal	
210	220
LysThrGlyValGlyAlaPheMetLysAspLeuAsnAspAlaLysAlaLeuAlaLysAla	
230	240
MetValAspIleGlyAsnArgValGlyArgLysThrMetAlaIleIleSerAspMetSer	

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[式6]

[Formula 6]

250	260
GlnProLeuGlyTyrAlaIleGlyAsnAlaLeuGluValLysGluAlaIleAspThrLeu	
270	280
LysGlyGluGlyProGluAspPheGlnGluLeuCysLeuValLeuGlySerHisMetVal	
290	300
TyrLeuAlaGluLysAlaSerSerLeuGluGluAlaArgHisMetLeuGluLysAlaMet	
310	320
LysAspGlySerAlaLeuGlnThrPheLysThrPheLeuAlaAlaGlnGlyGlyAspAla	
330	340
SerValValAspAspProSerLysLeuProGlnAlaLysTyrIleIleGluLeuGluAla	
350	360
LysGluAspGlyTyrValSerGluIleValAlaAspAlaValGlyThrAlaAlaMetTrp	
370	380
LeuGlyAlaGlyArgAlaThrLysGluSerThrIleAspLeuAlaValGlyLeuValLeu	
390	400
ArgLysLysValGlyAspAlaValLysLysGlyGluSerLeuValThrIleTyrSerAsn	
410	420
ArgGluGlnValAspAspValLysGlnLysLeuTyrGluAsnIleArgIleSerAlaThr	
430	
ProValGlnAlaProThrLeuIleTyrAspLysIleSer	

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[0013]

Further, the present invention relates to a recombinant vector prepared by integration of a DNA molecule containing the above nucleoside-phosphorylase-structured gene in the downstream of expression-controlling signal of the vector capable of replication in a cell, to a cultivated product containing microbe biomass of microorganisms retaining a nucleoside-phosphorylase derived from thermophilic bacteria in which the relevant enzyme has been generated by cultivation of a transformant transformed by the recombinant vector, and to microbe biomass of microorganisms retaining a nucleoside.

phosphorylase derived from thermophilic bacteria which has been isolated from the cultivated product, or to a processed product thereof.

[0014]

Furthermore, the present invention relates to a process for preparation of nucleoside characterized in that, in the process for preparation of nucleoside by a reaction of a base donor, a sugar-residue donor and a phosphoric acid donor using an enzyme preparation which contains nucleoside-phosphorylase to form N-glicoside linkages between the base segment of the base donor and the sugar segment of the sugar residue donor, the above cultivated product or the above microbe biomass of microorganisms or a processed product thereof is used as the enzyme preparation containing a nucleoside-phosphorylase.

[0015]

The present invention is described below in details. The following terms in this specification are used respectively with the definition shown below. The expression "derived from thermophilic bacteria belonging to bacillus genus" has a meaning of substantial identicalness of the base sequence of a DNA molecule with that of a gene of thermophilic bacteria belonging to bacillus genus but does not necessarily mean that the DNA fragment in the present invention is restricted to an extracted product from thermophilic bacteria belonging to bacillus genus. The phrase "the base sequence is substantially identical" means that, so far as the genetic information as a nucleoside-phosphorylase is maintained, substitution, default and/or addition of several unitary nucleotide (base) may exist.

[0016]

1. DNA molecules containing a nucleoside-phosphorylase-structured gene

The saying "DNA molecule containing a nucleoside-phosphorylase-structured gene derived from thermophilic bacteria belonging to bacillus genus" denotes those containing nucleoside-phosphorylase-structured gene coding the amino acid sequence of the above Formula (I) and/or Formula (II) and the specific base sequence thereof is not restricted in particular. For instance, it is possible to exemplify the DNA molecules specified in the restriction enzyme map shown in Figure 1, more specifically the DNA molecule containing the purine nucleoside-phosphorylase-structured gene, can be exemplified by that cut off at NcoI and HpaI, the DNA molecule containing the pyrimidine nucleoside-phosphorylase-structured gene can be exemplified by that cut off at PstI and HincII and the DNA molecule containing the gene structured by both purine nucleoside-phosphorylase and pyrimidine nucleoside-phosphorylase can be exemplified by that cut off at NcoI and EcoRI, respectively.

[0017]

Such DNA molecules contain an SD sequence at least in the upstream of structural gene in addition to the structural gene of nucleoside-phosphorylase so that they serve the object of present invention in respect of that they notably increase the produced amount of nucleoside-phosphorylase in comparison with the case of using DNA molecules containing only the structural gene. Accordingly, it is essential to use, in the



process of present invention, the DNA molecules containing a base sequence within the range at least from the SD sequence to the ending codon shown in Figures 2~4. In the cases of preparing both enzymes, purine nucleoside-phosphorylase and pyrimidine nucleoside-phosphorylase, it is sufficient to use those the DNA molecules of which include at least one SD sequence in the upstream of structural gene thereof.

[0018]

In Figure 1, "punA" indicates a gene coding the purine nucleoside-phosphorylase (822 bp, coding a polypeptide of molecular weight 29,637 consisting of 274 amino acids) and "pyn" indicates a gene coding the pyrimidine nucleoside-phosphorylase (1298 bp, coding a polypeptide of molecular weight 46,271 consisting of 433 amino acids). In the analysis by the inventors, both of the genes are forming a cluster respectively and have a ribosome binding site which is necessary for beginning translation but have no promoter structure of thermophilic bacteria and no promoter structure capable of functioning by *Escherichia coli* in the 5' upstream in the proximity thereof.

[0019]

Such DNA molecules can be subjected to cloning from thermophilic bacteria belonging to bacillus genus taking the nucleoside-decomposing activity at high temperatures as the index as seen in the above Yamanouchi's method (Japanese Patent Laid-Open Publication Hei 4-4882) or the like. Otherwise, it is possible to adopt a method, as conventionally well applied, in which a known method is used for determination of the partial

amino acid sequence such as the amino terminals of purified nucleoside-phosphorylase derived from thermophilic bacteria belonging to bacillus genus, or the sequence in part of the amino acid sequence of the above Formula (I) and/or Formula (II) is referred to, an oligonucleotide corresponding thereto is synthesized, and a DNA fragment containing a gene coding the nucleoside-phosphorylase is chosen from the gene bank of thermophilic bacteria belonging to bacillus genus using such an oligonucleotide as a probe. Although the host used for cloning is not restricted, it is appropriate to use *Escherichia coli* as the host in view of the operableness and the convenience. Further, there is no trouble also in chemical synthesis with use of an ordinary DNA synthesizer referring to Figures 2~4.

[0020]

Usually, even if these fragments are cloned on, e.g., a plasmid vector, such DNA fragments have no promoter or, though having a promoter, efficient functioning in heterogeneous microorganisms cannot be expected in many cases so that it is considered that high expression of coded gene is not induced as usual. Further, in the event of being provided with an extra DNA other than the coding region, the expression thereof may sometimes happen even by read through transcription from the promoter of another gene existing on the plasmid vector as an unfavorable event. Therefore, it is necessary for incarnating high expression of the gene as the object to analyze the base sequence of cloned DNA fragment to specify the coding region of the gene for preparing the recombinant expression vector in which the expression controlling signals (signals for beginning

of transcription and beginning of translation) are combined in the 5' upstream thereof so as to obtain possibility of high expression of the gene in the microbe biomass of microorganisms correspondingly to the host microorganisms. Determination of the DNA base sequence can be made by the ordinary method as exemplified by the possibility of conducting as application of Maxam-Gilbert method (Methods in Enzymology, 65, 499 (1980)), the dideoxy chain terminator method (Methods in Enzymology, 101, 20 (1983)) or the like.

[0021]

## 2. Recombinant vector

It is preferable to use, as the expression controlling signal used for expression of a large amount of nucleoside-phosphorylase genes in heterogeneous microorganisms, powerful signals for transcription beginning as well as for translation beginning capable of being artificially controlled which make great stride in increase of the expression amount of the nucleoside-phosphorylase genes. Such transcription beginning signals can be exemplified by expression controlling signals for genes such as lac promoter, trp promoter, tac promoter (Proc. Natl. Acad. Sci. USA., 80, 21 (1983); Gene, 20, 231 (1982)), trc promoter (J. Biol. Chem., 260, 3539 (1985)) etc. in the case of using Escherichia coli as the host and by glyceraldehyde-3-phosphate-dehydrogenase (J. Biol. Chem., 254, 2078 (1980)), depressive acidic phosphatase (Nucl. Acids Res., 11, 1657 (1983)) etc. in the case of using an yeast as the host.

[0022]

Although it is possible to use various plasmid

vectors, phage vectors and the like as the vector, it is preferable to use a plasmid vector being replicable in the microbe biomass, having a suitable drug resistant marker and a specific scission site of restriction enzyme and having a high copy number in the microbe biomass. In the case of specifically using *Escherichia coli* as the host, it is possible to exemplify by pBR322 (Gene, 2, 95 (1975)); pUC18, pUC19 (Gene, 33, 103 (1985)) and so forth. Further, in the case of using an yeast as the host, it is possible to exemplify by YEp13 (ATCC 37115), YEp24 (ATCC37051) and so forth. The methods for preparation of nucleoside-phosphorylase-structured gene, binding of a gene after cloning with an expression controlling signal etc. are well known technology for the technicians in general, particularly for the technicians belonging to the fields of molecular biology or genetic engineering so that it can be practiced depending on the method exemplified by that described in "Molecular Cloning" (edited by Maniatis et al., Cold Spring Harbor, New York (1982)).

[0023]

### 3. Preparation and cultivation of transformant

Transformation of microorganisms is carried out using the prepared recombinant vector. The microorganisms as the host is not restrictive in particular with a proviso of securing high safety and easy handling. For example, it is possible to use microorganisms conventionally used in DNA recombining operations such as *Escherichia coli*, yeast and the like. Among them, *Escherichia coli* is advantageous in vies of handling as well as of synthesis of nucleoside analogues as shown by the usefulness

of, for example, K12 strain, C600 microbe, JM105 microbe, JM109 microbe and the like used in recombinant DNA experiments. The method for subjecting microorganisms to transformation may be appropriately chosen from many methods previously reported correspondingly to the microorganisms used as the host. In the case of using *Escherichia coli*, for example, as the host, it is possible to subject *Escherichia coli* to transformation by the method of introducing a plasmid into the microbe biomass at a low temperature after processing by calcium chloride (J. Mol. Biol., 53, 159 (1970)). In the case of using an yeast as the host, it is possible to apply the processes such as the protoplast method (Proc. Natl. Acad. Sci. USA, 75, 1929 (1978)), the alkali metal treating method (J. Bacteriol., 153, 163 (1983)) and so forth.

[0024]

The obtained transformants are subjected to proliferation in a medium in which the relevant microorganisms can be multiplied and then further to cultivation by inducing of expression of the cloned nucleoside-phosphorylase genes until a large amount of the enzyme is accumulated in the microbe biomass. It is sufficient to carry out cultivation of the transformant by application of an ordinary method with use of a medium containing nutrient sources such as carbon sources, nitrogen sources and the like required for proliferation of the relevant microorganisms. For instance, in the case of using *Escherichia coli* as the host, it is possible to carry out cultivation using a cultivation medium conventionally used for cultivation of *Escherichia coli* such as 2xYT medium (Methods in

Enzymology, 100, 20 (1983)), LB medium, M9CA medium (Molecular Cloning, mentioned before) and the like at 20~40 °C of the cultivation temperature with aeration or stirring according to necessity. In the case of using plasmid as the vector, cultivation is carried out by compounding the cultivation medium with an appropriate amount of a drug of suitable antibiotic (such as ampicillin, tetracycline etc. corresponding to the drug-resistant marker of plasmid) in order to prevent falling down of the plasmid during cultivation.

[0025]

In the cases where induction of expression is required for nucleoside-phosphorylase gene in the course of cultivation, expression of the relevant gene is induced by the method ordinarily used for the used promoter. For instance, in the cases of using lac promoter or tac promoter, an appropriate amount of isopropyl- $\beta$ -D-thiogalactopyranosid (abbreviated as IPTG, hereinafter) as an expression inducing agent is added in the middle course of cultivation. In the cases where the used promoter has transcription activity as the constitution, addition of any expression inducing agent is not required in particular. After expression of the nucleoside-phosphorylase has been induced, cultivation is continued for further several hours for accumulation of a large amount of the gene product in the microbe biomass giving a cultivated product as the enzyme source. The cultivated product thus obtained can be used as an enzyme source for synthesis of a nucleoside analogue.

[0026]

4. Cultivated microbe biomass and processed product thereof

The recombinant bacteria are subjected to recovery of the microbe biomass after cultivation by any treatment such as membrane separation, centrifugal separation or the like. In the cases of using the cultivated microbe biomass as an enzyme source, the collected microbe biomass can be directly used in synthesis of a nucleoside analogue by suspending the collected microbe biomass in a suitable buffer solution. In order to carry out synthesis of an enzymic nucleoside analogue, it is sufficient to prepare the nucleoside-phosphorylase produced from the recovered microbe biomass. For instance, a cell-free extract solution is obtained from the recovered microbe biomass by suspending in a suitable buffer solution with subsequent crushing of the microbe biomass by a physical measure such as ultrasonic treatment, French press treatment or the like or bacteriolysis such as lysozyme treatment succeeded by removal of the residue of microbe biomass by centrifugation. Since the enzyme exists in excess in the cell-free extract solution, it is also possible to use the cell-free extract solution per se as a preparation. Even in the cases where further refining is required, it is possible to prepare a highly refined enzyme preparation suitable for synthesis of a nucleoside analogue by application of a simple means conventionally used for refining of enzymes such as heat treatment, salting out treatment by ammonium sulfate, dialysis treatment, solvent treatment by, e.g., ethanol, various types of chromatographic treatment and so forth as single application or as a combination of at most 2 kinds of treatments. In the cases of using *Escherichia coli* as the host microorganisms, almost whole of the protein derived

from *Escherichia coli* is denatured by subjecting the cell-free extract solution to heat treatment (at 60~80 °C for 1~10 minutes) to allow removal simply by operation of centrifuge so that it is possible to refine an enzyme with very high efficiency.

[0027]

#### 5. Synthesis of nucleoside analogue

The synthesis of nucleoside analogue according to the present invention is characterized by the use of above cultivated product, the above cultivated microbe biomass or the processed product thereof and other conditions or procedures are satisfied by following the known method (refer to, for example, International Patent Laid-Open Publication WO90/10080). That is to say, it is possible to practice by setting up the optimum condition of the used enzyme depending on a preliminary test and by carrying out the reaction of the raw compound with the cultivated product, the cultivated microbe biomass or the processed product thereof under this set condition. More specifically, it is possible to carry out by the reaction under the set condition of reaction temperature appropriately selected from 20~95 °C and of pH for reaction appropriately selected from 3~10 using an appropriate buffer solution containing an appropriate amount of inorganic phosphoric acid compounded with nucleoside as the sugar-residue donor and with, e.g., a base analogue as the base donor. The synthesized nucleoside analogue can be purified after the end of reaction, by application of an appropriate combination of the methods usually used as the purification method for substances relating to nucleic acid.



[0028]

[Effect of the invention]

According to the present invention, the above-mentioned problems in the prior art have been solved and it has been made possible to synthesize efficiently nucleoside analogues using a nucleoside-phosphorylase derived from thermophilic bacteria belonging to bacillus genus. Namely, the present invention provides the following advantages.

(a) It is made possible to synthesize efficiently a nucleoside analogue accompanied with few side reactions using a microbe biomass as the enzyme source. In the cases of synthesizing a nucleoside analogue using a microbe biomass of microorganisms belonging to bacillus genus, the side reaction proceeds even in the reactions at a high temperature since the substrates other than nucleoside-phosphorylase or product-decomposing enzymes have thermotolerance. However, almost all of enzymes derived from *Escherichia coli* are inactivated in the case of conducting the synthesizing reaction at a high temperature using *Escherichia coli* as the host microorganisms getting rid of almost all of the side reactions. Further, the preceding problem of autolysis is solved by using *Escherichia coli* as the host microorganisms. Also, since mass production of nucleoside-phosphorylase derived from thermophilic bacteria is carried out in the recombinant bacteria, use of a small amount of microbe biomass is sufficient in use for the synthesizing reaction so that it is made possible to efficiently synthesize in view of economy.

[0029]

(b) Preparation of a large amount of enzyme is made convenient so that it is made possible to put enzymic synthesis of a nucleoside analogue into practical application. Although it has been extremely difficult in the prior art to prepare nucleoside-phosphorylase from thermophilic bacteria belonging to bacillus genus, preparation thereof in a large amount has been extremely convenient by mass production of the enzyme as application of the recombinant DNA technique. For instance, the enzyme can be extracted in a high yield through a simple treatment such as lysozyme processing or ultrasonic processing by using Escherichia coli as the host microorganism. Also, since the enzyme has thermotolerance, it is possible to remove the enzyme of Escherichia coli participating in the side reaction by subjecting to heat treatment for inactivation and it is also made possible to prepare an enzyme preparation having high specific activity through a convenient operation. In the case of synthesizing a nucleoside analogue using both enzymes including purine nucleoside-phosphorylase and pyrimidine nucleoside-phosphorylase, there may be a phenomenon of excluding efficient reaction due to the rate determination by the reaction in which either one of both enzymes participates. However, purine nucleoside-phosphorylase and pyrimidine nucleoside-phosphorylase can be produced from the thermophilic bacteria only in a constant proportion and it has been extremely difficult to change the proportion. However, it is made possible to carry out efficient synthesis of a nucleoside analogue by separately producing both enzymes through the recombinant DNA technique for modulating the proportion. As

seen herein, the present invention serves in practical application of efficient production of nucleoside analogues so that an extreme benefit in industry is obtained.

[0030]

[Examples]

Specific explanation is given below in relation to nucleoside-phosphorylase derived from *Bacillus stearothermophilus* TH6-2 strain (deposition in Bi-Ko-Ken, No. 2758) with reference to examples. Also, "Molecular Cloning" (mentioned before) was applied to every item in the examples including preparation of DNA, scission by the restriction enzyme, DNA linking by T4 DNA ligase and transforming process of *Escherichia coli*. Further, all of various restriction enzymes, T4 DNA ligase, plasmid vectors pUC118 and pUC119, pPstI linker and delattion kit for kilosequence have been obtained from Takara Shuzo K.K.

[0031]

Example 1: Determination of DNA base sequence of DNA molecules containing thermophilic bacteria nucleoside-phosphorylase-structured gene

From the DNA fragment of 4.6 kb coding purine nucleoside-phosphorylase and pyrimidine nucleoside phosphorylase derived from thermophilic bacteria *Bacillus stearothermophilus* TH6-2 strain previously prepared by Yamanouchi et al. (refer to Figure 1, Japanese Patent Laid-Open Publication Hei 4-4882, *Escherichia coli* K12 strain retaining pUC119 plasmid in which this fragment is inserted, *Escherichia coli* KY-2, has been deposited in Kogyo Gijutsuin (Agency of Industrial Science and

Technology), Biseibutsu Kogyo Gijutsu Kenkyusho (Research Institute for Industrial Technology of Microorganisms) on January 16, 1990 and given with the deposition number "Bi-Ko-Ken Microbe Deposition No. 11197), SacI-EcoRV DNA fragment considered as containing purine nucleoside-phosphorylase-structured gene was sub-cloned to pUC118 and pUC119. The recombinant plasmid was prepared in various clones having distinctive chain length by falling off of a part of the relevant inserted sites using a deletion kit for kilosequence following the method of literature (Gene, 28, 351 (1984)). Determination of the DNA base sequence was carried out by the dideoxy chain terminator method (Science, 214, 1295 (1981)) for inserted fragments of various clones thus obtained. Consequently, the DNA sequence of purine nucleoside-phosphorylase-structured gene was obtained as shown in Figure 2. This base sequence is coding a polypeptide of molecular weight 29,637 consisting of 274 amino acids started by Met. The amino acid sequence of 20 amino terminals of this peptide perfectly coincided with that of the refined and isolated purine nucleoside-phosphorylase.

[0032]

Then, with respect to PstI-EcoRI DNA fragment of 2.8 kb considered as containing the pyrimidine nucleoside-phosphorylase-structured gene, pUC118 and pUC119 were subjected to shot gun-subcloning using various restriction enzymes for carrying out determination of the base sequence thereof by the dideoxy chain terminator method similarly to the above. Consequently, the pyrimidine nucleoside-phosphorylase-structured

gene shown in Figure 3 and Figure 4 was obtained. This base sequence is 1,298 bp and is coding a polypeptide of molecular weight 46,271 consisting of 433 amino acids. The amino acid sequence of 10 amino terminals in this peptide perfectly coincided with that of refined and isolated thermophilic bacteria, pyrimidine nucleoside-phosphorylase.

[0033]

Example 2: Preparation of recombinant vector for high expression of thermophilic bacteria nucleoside-phosphorylase

The following method was applied to preparation of recombinant vector pTrc-nunA for producing purine nucleoside-phosphorylase (refer to Figure 5).

That is to say, after scission of a plasmid vector pTrc99A (Gene, 69, 301 (1988), obtained from Pharmacia company) using a restriction enzymes NcoI and SmaI, NcoI-HpaI DNA fragment containing purine nucleoside-phosphorylase-structured gene and SD sequence was jointed with the above broken fragment of the plasmid vector pTrc99A using T4 DNA ligase and then Escherichia coli JM105 strain was subjected to transformation using the solution of above scission reaction. The above obtained ampicillin-resistant transformant gave a recombinant vector Trc-punA in which an SD sequence and purine nucleoside-phosphorylase-structured gene were inserted directly behind the trc promoter for expression of the pTc99A.

[0034]

Then, a recombinant vector pTrc-pyn for high expression of pyrimidine nucleoside-phosphorylase was prepared (refer to Figure 6). That is, the above DNA fragment of 4.6 kb

was partly decomposed using a restriction enzyme HincII and the product thereof was further linked with pPstI linker using T4 DNA ligase. The reaction product was further broken with a restriction enzyme PstI for preparing PstI DNA fragment of 2.2 kb containing pyrimidine nucleoside-phosphorylase-structured gene and SD sequence thereof. This DNA fragment and pTrc99A broken with the restriction enzyme PstI were subjected to linking reaction using T4 DNA ligase and further Escherichia coli JM105 strain was subjected to transformation using the above reaction solution. The obtained ampicillin tolerant transformant gave a recombinant vector pTrc-pyn in which the SD sequence and pyrimidine nucleoside-phosphorylase-structured gene were inserted in the scission site directly behind the trc promoter of pTrc99A coinciding with the transcribing direction from trc promoter.

[0035]

Further, a vector pTrc-NE for producing both enzymes of thermophilic bacteria, purine nucleoside-phosphorylase and pyrimidine nucleoside-phosphorylase, was prepared (refer to Figure 7). That is to say, from the above DNA fragment of 4.6 kb, NcoI-EcoRI DNA fragment containing structural genes of both enzymes and the respective SD sequences thereof was linked with Trc99A broken by the restriction enzymes NcoI and EcoRI using T4 DNA ligase and Escherichia coli JM109 strain was transformed using the solution of linking reaction. The obtained ampicillin-tolerant transformant gave a recombinant vector pTrc-NE in which the SD sequences and the structural genes of purine nucleoside-phosphorylase and of pyrimidine nucleoside-phosphorylase were inserted in the scission site directly behind the trc promoter of pTrc99A coinciding with the transcribing direction from trc promoter.



°C carried out following Yamanouchi's method (Japanese Patent Laid-Open Publication Hei 4-4882).

[0037]

[Table 1]

bacteria/ plasmid	purine nucleoside decomposing activity (units/mg protein)	pyrimidine nucleoside decomposing activity (units/mg ptotein)**
JM105/pTrc99Δ	0.9	1.1
JM105/pUC119-PYR2	17.1	11.6
JM105/pTrc-punΔ	134.3	(not determined)
JM105/pTrc-pyn	(not determined)	100.3
JM109/pTrc-NE	134.7	66.0

\*1 unit = 1 μ mole Hypoxanthine-production/min. at 70 °C

\*\*1 unit = 1 μ mole Uracil-production/min. at 70 °C

[0038]

As shown in Table 1, identification has been attained with respect to the prepared transformant retaining the recombinant vector for the nucleoside phosphorylase activity having 100 fold or more of that of control bacteria (Escherichia coli JM105 retaining pTrc99A) at high temperatures. Also, it has been ascertained that the transformant (bacteria retaining pTrc-NE) prepared in the present invention can produce 6~8 fold of nucleoside-phosphorylase as compared with the transformant (bacteria retraining pUC119-PYR2) prepared by the prior art method (Japanese Patent Laid-Open Publication Hei 4-4882).



Further, the productivity of this transformant corresponds to about 8 fold of that of the thermophilic bacteria *Bacillus stearothermophilus* TH6-2 strain which is the original strain. Identical property with that of the enzyme derived from thermophilic bacteria was observed for the nucleoside phosphorylase prepared from the transformant of *Escherichia coli* retaining these recombinant vectors.

[0039]

Example 4: Synthesis of nucleoside analogue (ribavirin) using cultured microbe biomass

A cultured biomass was collected from 10 ml of culture medium of *Escherichia coli* JM109 strain retaining pTrc-NE obtained by the method similar to that in Example 3 by centrifugation and was suspended in 1 ml of physiological saline. To this biomass suspension, 40 mM 1,2,4-triazole-3-carboxamide (hereinafter abbreviated as "triazole") as a base donor and 9 ml of 40 mM phosphoric acid buffer solution (pH 6.0) containing 60 mM of uridine as a sugar residue donor were added to be reacted at 45 °C for 1 hour for synthesis of ribavirin. The generated ribavirin was subjected to determination of the yield of ribavirin by HPLC following the method taught in a document (International Patent Laid-Open Publication WO90/10080) giving a result that ribavirin was synthesized in an amount of 92 % as the ratio to triazole. Generation of any byproduct was not observed.

[0040]

Example 5: Synthesis of nucleoside analogue (ribavirin) using culture medium of microbe biomass

To 10 ml of phosphoric acid buffer solution of the constitution identical with that in Example 4 containing triazole uridine, 186  $\mu$ l of the biomass extract medium derived from the transformant JM109/pTrc-NE prepared in Example 3 was added (end concentration; purine nucleoside-phosphorylase 10 unit/ml; pyrimidine nucleoside-phosphorylase 5 unit/ml) to be subjected to a reaction at 50 °C for 8 hours. The yield of ribavirin was determined by the method similar to that in Example 4 giving a confirmation that ribavirin was synthesized in an amount of 90 % as the ratio to triazole. Generation of any byproduct was not observed similarly to the above.

[0041]

[Brief explanation of the drawing]

[Figure 1]

Figure 1 is illustrating the restriction enzyme map of 4.8 kb SacI-EcoRI DNA fragment containing purine nucleoside-phosphorylase- and pyrimidine nucleoside-phosphorylase-structured gene derived from thermophilic bacteria *Bacillus stearothermophilus* TH6-1 strain.

[Figure 2]

Figure 2 is illustrating the base sequence of DNA fragment containing purine nucleoside-phosphorylase derived from thermophilic bacteria *Bacillus stearothermophilus* TH6-1 strain. In the figure, S.D. indicates the SD sequence, Met indicates the codon for translation beginning of purine nucleoside-phosphorylase-structured gene, "stop" indicates the stopping codon thereof, and "Met of qppyn" indicates the codon for translation beginning of pyrimidine nucleoside-phosphorylase-

structured gene, respectively.

[Figure 3]

Figure 3 is illustrating the base sequence of DNA fragment containing pyrimidine nucleoside-phosphorylase-structured gene derived from thermophilic bacteria *Bacillus stearothermophilus* TH6-1 strain. In the figure, S.D. indicates the SD sequence, Met indicates the codon for translation beginning of pyrimidine nucleoside-phosphorylase-structured gene, and "stop of gppunA" indicates the ending position of purine nucleoside-phosphorylase-structured gene, respectively.

[Figure 4]

Figure 4 is illustrating the base sequence of DNA fragment containing pyrimidine nucleoside-phosphorylase-structured gene derived from thermophilic bacteria *Bacillus stearothermophilus* TH6-1 strain. In the figure, "stop" indicates the stopping codon thereof. The base sequences shown in Figure 3 and Figure 4 are a series of continued base sequence.

[Figure 5]

Figure 5 is illustrating the constructing method for the recombinant plasmid vector pTrc-punA.

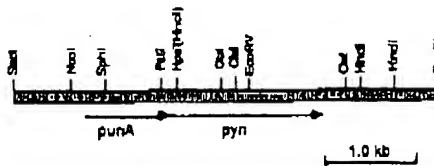
[Figure 6]

Figure 6 is illustrating the constructing method for the recombinant plasmid vector pTrc-pyn.

[Figure 7]

Figure 7 is illustrating the constructing method of the recombinant plasmid vector pTrc-NE.

[Figure 1]



(14)

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[Figure 2]  
(2)

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      10      20      30      40      50      60
GCATCGAAAC GACCCTGTTC ATCAGGGGAC AGATCATACA CGCGAATACC TACCGCTTCT
NcoI
      70      80      90      100     110     120
TGTGTATAGC CCAAGCATGA ACGGAGGTAA ACAACTTCCA TTCCGTGAAA CCTTTGCACA

      130     140     150     160     170     180
TGTAGGTGCA ACGATTGCAG AAAACTTTGC TGTGAAGATG CCAAAATACG GAACAAGTTT

      190     200     210     220     230     240
TTTACAAGAG TTGAAATAAG GAGCGTGAAC ATATGAATCG AACAGCTATT GAACAACGGG
S.D. Met
      250     260     270     280     290     300
CACAAATTTT AAAAGAAAAG TTTCCAACCT CACCGCAAAAT CGGCTTAATT CTTGGCTCTG

      310     320     330     340     350     360
GCTTAGGTGT GTTGGCCGAT GAGATTGAAC AAGCCATTAA AATTCGGTAC AGCGACATTG

      370     380     390     400     410     420
CGAATTTCCC TGTATCGACG GTTGAAGGGC ATGCCGGTCA GCTCGTATAC GGTGAGTTAG

      430     440     450     460     470     480
AAGGGGCAAC AGTAGTCGTT ATGCAAGGGC GGTTCATTA TTACGAAGGA TACAGCTTCG

      490     500     510     520     530     540
ATAAGGTAAC GTTCCCTGTG CGCGTGATGA AAGCTCTCGG TGTGAGGCAG TTAATTGTCA

      550     560     570     580     590     600
CAAAATCGGC AGGTGGTGTG AATGAATCGT TTGAACCGGG CGATTTAATG ATTATTTTCA

      610     620     630     640     650     660
ATCATATTAA TAACATGGGC GCGAATCGGC TTATCGCTCC GAATGATICT GCAGTTGGGG

      670     680     690     700     710     720
TCCGCTTCCC AGACATGTCT GAAACATATA GTAAACGACT TCGTCAACTT GCCAAAGATG

      730     740     750     760     770     780
TAGCAAAACG CATCGGTTTA CGTGTGCCCG AAGGTGTGTA TGTGCGCAAT ACGGGTCCAC

      790     800     810     820     830     840
CGTATGAAAC GCGGGCAGAA ATTCGTATGA TTCGTGTCAT CGCTGCGGAT GCTGTTGGTA

      850     860     870     880     890     900
TGTCAACGGT GCCTGAAGTG ATCGTTGCCC GCCATCCCCG AATGGAAGTG CTCGGTATTT

      910     920     930     940     950     960
CGTGTATTTT GAATATGGCT GCAGGTATTT TACATCAGCC GCTTACCCAT CATCAACTCA

      970     980     990     1000    1010    1020
TCGAAACGAC CGAAAAAGTA AAAGCTGACT TTTTACGATT TGTGAAGGCC ATCGTACCCA

      1030    1040    1050    1060    1070    1080
ACATCGCCAA AAATTAAACC AGAAGGTGAA CGACGATCAG AATGGTCCAT TTAATTGAGA
Stop Met of gpyn
      1090    1100    1110
AAAAACGTGA TGCTCATGCC TTAAC
HpaI

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(15)

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(Figure 3)

[図3]

10	20	30	40	50	60
CTGCACGTAT	TTTAGATCAG	CCGCTTACGC	ATCATGAAGT	GATCGAAACG	ACGGAAAAAC
PstI					
70	80	90	100	110	120
TAAAAGCTGA	CTTTTACGA	TTTGTGAAGG	CGATCGTACC	CAACATGCCG	AAAAATTAAA
				stop of gppuPA	
130	140	150	160	170	180
CGACAAGGTC	AACGACGATG	AGAATGGTCG	ATTTAATTGA	AAAAAACCGT	GATGGTCATC
S.D.	Met				
190	200	210	220	230	240
CGTTAACGAA	AGAAGAAATT	CAGTTTATTA	TTCAAGCTTA	CACAAAAGGC	GATATTCCCTG
250	260	270	280	290	300
ATTATCAAAT	GAGCGCATT	GCGATGGCGA	TTTTTTTCCG	CGGCATCAAT	GAAGAAGAGA
310	320	330	340	350	360
CAGCGGAATT	GACGATGGCG	ATGGTGCATT	CAGGGGATAC	GATCGACCTT	TCGGCAATTC
370	380	390	400	410	420
AAGGAATTAA	AGTAGACAAA	CATTCAACGG	GCGGAGTGGG	CGATACAACA	ACGTTAGTGC
430	440	450	460	470	480
TTGGCCCTCT	TGTCGGCTCC	GTCGGTGTTC	CGGTTGCGAA	AATGTCTGGG	CGCGGCCTTC
490	500	510	520	530	540
GACATACGGG	TGGAACGATC	GACAAACTAG	AATCGGTGCC	AGGTTTTTCAC	GTTGAAATTA
550	560	570	580	590	600
CGAACGATGA	ATTTATCGAT	CTTGTCATA	AAAATAAAAT	TCCCGTTGTC	GGTCAGTCTG
610	620	630	640	650	660
GTAATTTCAC	GCCAGCGGAC	AAAAAGTTGT	ATGCGCTTCG	TGATGTGACG	GCAACGGTCA
670	680	690	700	710	720
ATAGCATTC	GTAAATTGCC	TCATCGATTA	TGAGCAAAAA	AATTGCCGCCA	GGGGCAGATC
730	740	750	760	770	780
CGATCGTACT	TGACGTAAAA	ACAGGTCTCG	GCGCGTTTAT	GAAACATTTA	AACGATGCAA
790	800	810	820	830	840
AAGCATTAGC	GAAAGCGATC	GTCGATATCG	GAAATCGGCT	TGGCCGTAAA	ACGATCGCAA
850	860	870	880	890	900
TTATTTCTGA	TATGAGCCAG	CCGCTTGCTT	ATGCCATTGG	AAATGCCCTT	GAAGTCAAAAG
910	920	930	940	950	960
AAGCGATTGA	TACGTAAAA	GGAGAAGGTC	CAGAAGATTT	CCAAGAGCTG	TGCTTAGTGC
970	980	990	1000	1010	1020
TTGCTAGCCA	CATGGTATAT	TTAGCGGAAA	AAGCATCTTC	GCTTGAAGAA	GCTCCTCATA
1030	1040	1050	1060	1070	1080
TGTTAGAAAA	AGCGATGAAA	GACGGTTCAG	CCCTTCAAAC	ATTTAAACCG	TTCTTAGCTG

(その1)

(16)

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(Figure 4)

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1090      1100      1110      1120      1130      1140
CCCAAGGTGG CGATGCATCT GTTCTCGATC ACCCAAGCAA ATTGCCGCAA GCAAAATATA

1150      1160      1170      1180      1190      1200
TTATTGAACT AGAAGCGAAA GAAGATGGAT ACGTATCCGA AATTGTGCGG GATGCCGTCC

1210      1220      1230      1240      1250      1260
CAACCGCGGC GATGTGGCTT GGTGCAGGGC GAGCGACGAA AGAATCAACG ATCGATTTAG

1270      1280      1290      1300      1310      1320
CTGTCCGTCT CGTGCTTCGC AAAAAAGTCG GCGATGCGGT GAAAAAAGGT GAATCGCTCC

1330      1340      1350      1360      1370      1380
TTACAATTTA CAGCAACCGT GAACAAGTGG ATGATGTAAA ACAAAAACTA TATGAAAACA

1390      1400      1410      1420      1430      1440
TTCGTATTTC AGCAACACCT GTTCAAGCTC CAACATTAAT TTACGATAAA ATTCGTAAC
                                STOP
1450      1460      1470      1480      1490      1500
CTGAAGGATT CATTCCTTCA GGTTTTTTTA TGTATAAAAA AATAAAAAAT GGAAAGGATG

1510      1520      1530      1540      1550      1560
GCAAGTATAA AGAAATGGAG GGTGGAGAT GAAGCGATTT TTTTGCATGT TTGCTATGGC

1570      1580      1590      1600      1610      1620
GTTTCTTTTT CTCCAAGCG TCGTTGTGCG CGCGGAACAG CCGAAAATTG AATTAGCACC

1630      1640      1650      1660      1670      1680
TGAGGCGCGA TCAGCAATTT TAATTGAGAG AGACACAGGG GCTGTTTTGT ATGAAAAAAA

1690      1700      1710      1720      1730      1740
TGCCCATGAG CCGCTTCCAC CAGCGAGCAT GACGAAAATT ATGACAATGC TTCTCATTAT

1750      1760      1770      1780      1790      1800
GGAAGCGATT GATCAAGGAA AGTTGAAGAT AGACGAGCGA GTGCGCGCAA GTGAATACCC

1810
TGCATCGAT
Clai

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(その2)

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【圖 5】

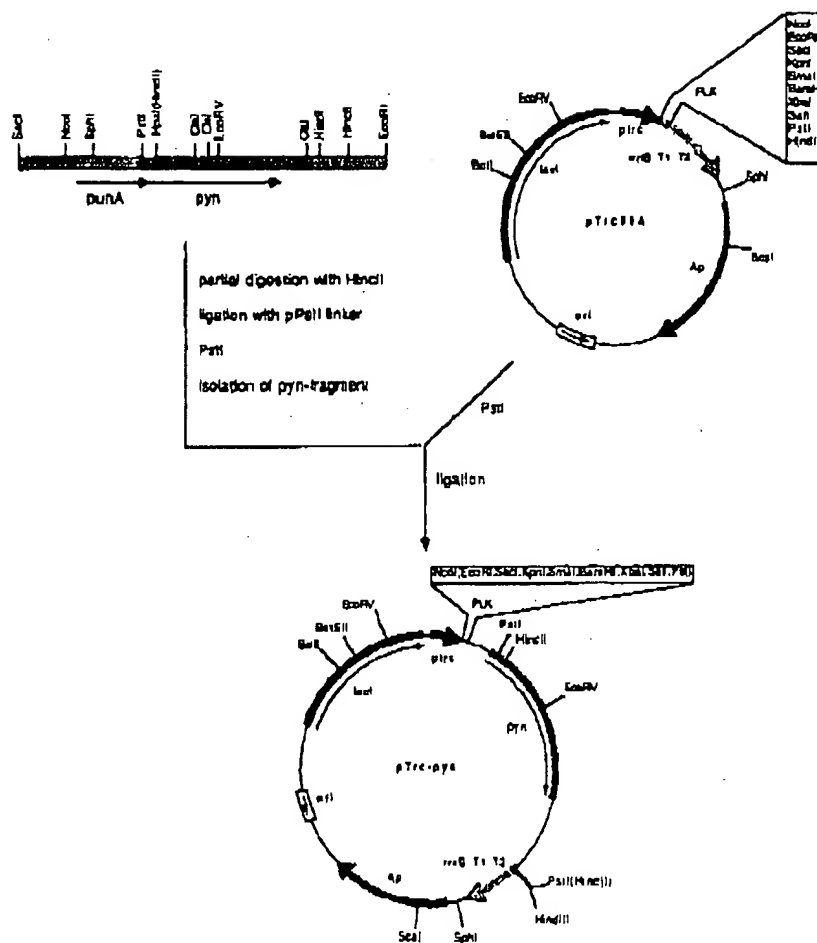




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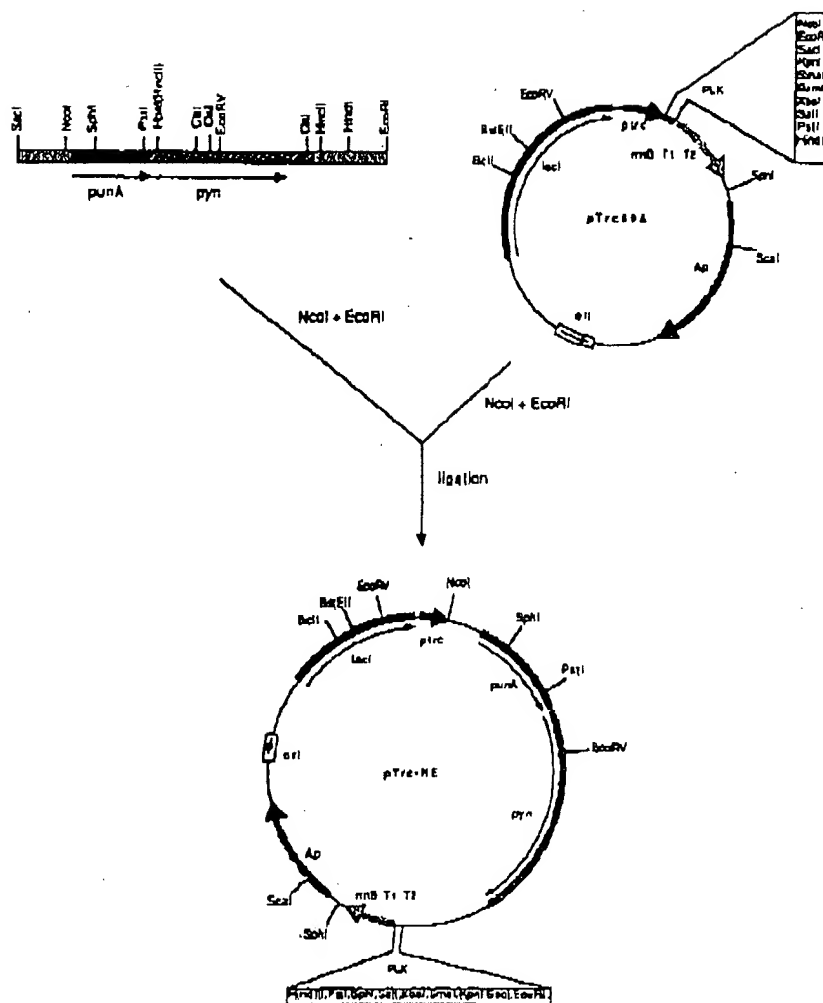
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[Figure 6]  
[図 6]



(19)

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[Figure 7]  
[図7]

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C 12 R 1:19)

(C 12 N 9/10

C 12 R 1:19)